NITROGEN FIXATION AND CELLULOSE DECOMPOSITION BY SOIL MICRO-ORGANISMS. II.

THE ASSOCIATION BETWEEN AZOTOBACTER AND FACULTATIVE-AEROBIC CELLULOSE-DECOMPOSERS.

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(One Text-figure.)

[Read 28th May, 1941.]

Introduction.

In a previous paper (Jensen, 1940*b*) it was shown that no fixation of nitrogen takes place in combined pure cultures of *Azotobacter* and "typical" aerobic cellulose-decomposing soil bacteria (*Cytophaga, Cellvibrio*, etc.), as well as fungi or actinomycetes, but that certain facultative aerobes which decompose the cellulose with formation of organic acids, enable *Azotobacter* to fix appreciable quantities of nitrogen. The present paper gives an account of the quantitative relationship between cellulose decomposition and nitrogen fixation under varying experimental conditions, and the nature of the organic breakdown products of cellulose that serve as energy material for the fixation.

Four strains of cellulose-decomposing bacteria were tested: three species of corynebacteria (*Cor.* 3 (*fimi*?), *Cor.* Va and *Cor.* Vb, described previously (Jensen, 1940*b*)), and an authentic strain of *Cellulomonas biazotea*.* The strain of *Az. chroococcum* used in the previous investigation was used throughout the experimental work, and occasionally *Az. vinelandii, Az. Beijerinckii* (?) and *Az. indicum* (the last kindly supplied by Dr. R. L. Starkey, New Jersey Agricultural Experiment Station, N.J., U.S.A.). The basal solution for the combined cultures, unless otherwise stated, contained: K_2HPO_4 0·1%, $MgSO_4$ 0·05%, NaCl 0·02%, $FeCl_3$ 0·01%, Na_2MoO_4 0·001%, besides varying amounts of $CaCO_3$ and yeast extract (of 10% dry yeast in tap water, autoclaved and filtered). Cellulose was supplied as filter paper—Whatman No. 1. Inoculum consisted of one loopful of a thin suspension of cells from young cultures on agar slopes. Nitrogen was determined by the Kjeldahl method as previously described (Jensen, 1940*a–b*), and soluble organic carbon by the method of Birkinshaw and Raistrick (1931). Residual cellulose was determined by filtration through Jena glass filter crucibles (No. 1–G–1), washing with dil. HCl and distilled water, and drying to constant weight at 98°C. It was found that both cellulose and nitrogen could, without any serious error, be determined in the same culture by removing the dried residue from the crucible and digesting it together with the combined filtrate and washings. Volatile acids were determined by distillation according to Duclaux, and lactic acid by the method of Friedemann *et al.* (1927).

EXPERIMENTAL.

A. *Nitrogen Fixation in Associated Cultures.*

Experiment 1: The four cellulose-decomposing bacteria were tested in combination with *Az. chroococcum*. The basal solution contained 2·0% yeast extract and 0·5% $CaCO_3$;

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* A strain of *Cell. rossica* (Kellerman), obtained from the National Collection of Type Cultures, Lister Institute, London, was also tested. This organism, however, was found to have lost its cellulose-decomposing power.

the first series, with *Cor.* 3, was carried out in 250 c.c. round flasks with 100 c.c. solution and 1·0 gm. filter paper, the others in 100 c.c. round flasks with 40 c.c. solution and 0·5 gm. filter paper. Table 1 gives the results.

TABLE 1.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *with Corynebacteria and* Cellulomonas biazotea.

Inoculum.	Incubation, Days.	Cellulose, gm. Per Culture.	Cellulose, gm. Loss.	Nitrogen, mgm. Per Culture.	Nitrogen, mgm. Gain.
I.					
Control	0	0·951		2·40 2·41 } 2·40	
Corynebacterium 3	48	(*a*) 0·797 (*b*) 0·822	0·154 0·129		
,, +*Azotobacter* ..	48	(*a*) 0·728 (*b*) 0·762 (*c*) 0·800	0·223 0·189 0·151	(*d*) 4·04 (*e*) 4·22 (*f*) 4·72	1·64 1·82 2·32
II.					
Control	0	0·478		2·66 2·72 } 2·69	
Azotobacter	35			2·65	(−0·04)
Corynebacterium Vb	35			2·80	(0·11)
,, Va	42			2·78	(0·09)
,, Vb+*Azotobacter* ..	35	(*a*) 0·251 (*b*) 0·238 (*c*) 0·235	0·227 0·240 0·243	(*d*) 6·30 (*e*) 6·68 (*f*) 5·26	3·61 3·99 2·57
,, Va+*Azotobacter* ..	42	(*a*) 0·332 (*b*) 0·330 (*c*) 0·328	0·146 0·148 0·150	(*d*) 4·31 (*e*) 4·48 (*f*) 4·41	1·62 1·79 1·72
III.					
Control	0	0·478		2·36 2·20 } 2·28	
Cellulomonas biazotea+*Azotobacter* ..	45	(*a*) 0·405 (*b*) 0·404	0·073 0·074	(*a*) 2·73 (*b*) 2·53	0·45 0·25

	Average Loss of Cellulose, gm.	Average Gain of N, mgm. Per Culture.	Average Gain of N, mgm. Per gm. of Cellulose Lost.
Azotobacter +*Corynebacteria* 3	0·188	1·93	*10·3*
,, + ,, Vb	0·237	3·39	*14·3*
,, + ,, Va	0·148	1·71	*11·5*
,, +*Cellulomonas biazotea*	0·074	0·35	*4·7*

The pure cultures of cellulose-decomposers absorb no nitrogen from the atmosphere, and *Azotobacter* does not grow in the absence of cellulose-decomposers (cf. Jensen, 1940*b*). The associated cultures show in all cases a strong cellulose decomposition and significant gains of nitrogen which, although varying considerably in replicate cultures, correspond to fixation of about 4 to 14 mgm. N per gm. of cellulose decomposed. This figure agrees perfectly with the findings (reviewed by Jensen, 1940*a*) of many previous investigators who have used crude cultures or natural populations of soil or decaying plant material. Both absolutely and relatively, *Cell. biazotea* appears the least and *Cor.* Vb the most efficient organism. The latter was therefore used in most of the subsequent experiments.

Experiment 2: Various other species of *Azotobacter* were tested; in the first series, *Cor.* 3 together with *Az. vinelandii*, in the second, *Cor.* Vb with *Az. vinelandii*, *Az. Beijerinckii* and *Az. indicum*. Cultures were grown in 100 c.c. round flasks with 0·5 gm. filter paper, 0·2 gm. $CaCO_3$ (not added to the cultures of *Az. indicum*) and 40 c.c. of basal solution which in the first series contained 0·01% peptone, and in the second 2·0% yeast extract. Table 2 shows that the efficiency of fixation in terms of nitrogen per gm. of cellulose lost is similar to that in the cultures of *Az. chroococcum*, although the actual gains are smaller in *Az. indicum*.

TABLE 2.

Nitrogen Fixation in Combined Cultures of Corynebacteria and Different Species of Azotobacter.

Inoculum.	Incubation, Days.	Cellulose, gm.		Nitrogen, mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Series I, peptone solution.					
Control, duplicate	0	0·478		1·22	
Corynebacterium 3	28	0·466	0·012	1·28	(0·06)
,, 3 + *Azotobacter vinelandii*	28	(*a*) 0·411	0·067	(*d*) 2·00	0·78
		(*b*) 0·428	0·050	(*e*) 1·97	0·75
		(*c*) 0·404	0·074	(*f*) 1·70	0·48
Series II, yeast extract solution.					
Control, duplicate	0	0·478		2·17	
,, single	46	0·477		2·21	(0·04)
Corynebacterium Vb	48	0·392	0·086	2·34	(0·17)
,, ,, + *Azotobacter vinelandii*	42	(*a*) 0·257	0·223	(*a*) 5·01	2·84
		(*b*) 0·271	0·207	(*b*) 4·67	2·50
		(*c*) 0·260	0·218	(*c*) 4·72	2·55
,, ,, + *Azotobacter Beijerinckii*	45	(*a*) 0·264	0·214	(*a*) 5·21	3·04
		(*b*) 0·275	0·203	(*b*) 4·59	2·42
		(*c*) 0·287	0·191	(*c*) 4·48	2·31
,, ,, + *Azotobacter indicum*	48	(*a*) 0·362	0·116	(*a*) 3·45	1·28
		(*b*) 0·351	0·127	(*b*) 3·52	1·35
		(*c*) 0·317	0·161	(*c*) 3·93	1·76

Average gain of nitrogen per gm. of cellulose lost by:
Corynebacterium 3 + *Azotobacter vinelandii*: 10·5 mgm.
,, Vb + ,, ,, : 12·2 mgm.
,, Vb + ,, *Beijerinckii*: 12·8 mgm.
,, Vb + ,, *indicum* : 10·8 mgm.

Experiment 3: The influence of temperature on the efficiency of fixation was tested. *Cor.* Vb and *Az. chroococcum* were grown in 100 c.c. round flasks with 0·5 gm. filter paper, 0·2 gm. $CaCO_3$, and 40 c.c. basal solution + 2·0% yeast extract, at 15°C. and 37°C. The results (Table 2) show no striking difference between the two temperatures, although the efficiency appears lower than at 28–30°C. (cf. Table 1). The hypothesis of Olsen (1932) that a low temperature of decomposition might lead to a more copious formation of organic by-products and consequently stronger nitrogen fixation, finds no support in these data.

Experiment 4: This was undertaken with the same organisms and the same medium as Exp. 3, but with incubation at 28–30°C. and analysis at three stages of growth. It appears from the results in Table 4 that the efficiency increases with the age, i.e., the nutrients for *Azotobacter* seem to be formed more abundantly in ageing cultures of the cellulose-decomposers.

TABLE 3.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and* Corynebacterium *Vb at Different Temperatures.*

Culture Series.		Incubation, Days.	Cellulose, gm.		Nitrogen, mgm.	
			Per Culture.	Loss.	Per Culture.	Gain.
Control (duplicate)		0	0·478		3·94	
15° C.	*Corynebacterium* Vb ..	42			3·88	(−0·06)
	,, ,, +*Azotobacter*	42	(*a*) 0·364	0·114	(*d*) 4·76	0·82
			(*b*) 0·380	0·098	(*e*) 4·44	0·50
			(*c*) 0·398	0·080	(*f*) 4·45	0·51
37° C.	*Corynebacterium* Vb ..	20			(*a*) 3·74	(−0·20)
					(*b*) 3·59	(−0·35)
	,, ,, +*Azotobacter*	20	(*a*) 0·319	0·159	(*d*) 5·06	1·12
			(*b*) 0·335	0·143	(*e*) 5·30	1·36
			(*c*) 0·309	0·169	(*f*) 5·17	1·23

Average gain of N per gm. of cellulose lost: 15° C.: 6·3 mgm.
37° C.: 7·9 mgm.

TABLE 4.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and* Corynebacterium *Vb at Different Stages of Growth.*

Inoculum.	Incubation, Days.	Cellulose, gm.		Nitrogen, mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Control (duplicate)	0	0·478		2·28	
Corynebacterium Vb	32	0·394	0·084	2·18	(−0·10)
	52	0·386	0·092	2·04	(−0·24)
,, ,, +*Azotobacter* ..	14	(*a*) 0·374	0·104	(*c*) 2·78	0·50
		(*b*) 0·377	0·101	(*d*) 3·06	0·78
	32	(*a*) 0·312	0·166	(*a*) 3·83	1·55
		(*b*) 0·308	0·170	(*b*) 3·88	1·60
	52	(*a*) 0·286	0·192	(*a*) 4·44	2·16
		(*b*) 0·254	0·224	(*b*) 4·63	2·35

Average gain of N per gm. of cellulose decomposed: 14 d.: 6·2 mgm.
32 d.: 9·4 mgm.
52 d.: 10·8 mgm.

Experiment 5: In all the previous experiments the filter paper was added to a liquid medium in a fairly deep layer. At the start of incubation a part of the paper protruded above the liquid, but with advancing decomposition it soon collapsed and was entirely submerged, so that most of the cellulose decomposition probably took place under a somewhat restricted access of oxygen, the tension of which would be further decreased by the vigorous growth of *Azotobacter* on the surface of the medium, where something approaching a true *Azotobacter*-pellicle was often noticeable. An experiment was therefore designed to test the nitrogen fixation when cellulose was being decomposed under strictly aerobic conditions; this was done by placing the filter paper on the surface of an agar medium. The first series was carried out with *Cor.* 3 and *Az. chroococcum* in 250 c.c. Erlenmeyer flasks containing 75 c.c. basal solution + 0·8% agar and 2·0% yeast extract; $FeCl_3$ was replaced by 0·02% $FeSO_4$, which was added separately in solution after melting of the agar, in order to prevent precipitation of the iron as phosphate. After the agar had set, 0·5 gm. finely cut filter paper and 0·2 gm. $CaCO_3$, sterilized separately, were distributed evenly over the agar surface, where the condensation water was just sufficient to moisten the paper thoroughly; two flasks received an extra 10 c.c. of sterile water, thus immersing the paper in a shallow layer of liquid. The second series was carried out with *Cor.* Vb and *Az. chroococcum* in 250 c.c. round flasks with 80 c.c. agar medium, otherwise as in the first series. The paper was rapidly attacked, but vigorous growth of *Azotobacter*, as indicated by pigment formation, was observed only where extra water had been added.

TABLE 5.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and Corynebacteria on Agar Surface.*

Inoculum.	Incubation, Days.	Cellulose, gm. Per Culture.	Cellulose, gm. Loss.	Nitrogen, mgm. Per Culture.	Nitrogen, mgm. Gain.
I.					
Control	0	0·478		3·93 3·88 } 3·91	
Azotobacter	42			3·78	(−0·13)
Corynebacterium 3	42			3·91	(0)
		(*a*) 0·323	0·155	(*d*) 4·10	0·19
,, 3 + *Azotobacter* ..	42	(*b*) 0·315	0·163	(*e*) 4·18	0·27
		(*c*) 0·320	0·158	(*f*) 4·41	0·50
,, 3 ,, +10 c.c. water	42			(*a*) 4·80	0·89
				(*b*) 4·69	0·78
II.					
Control	0	0·478		3·70 3·74 } 3·72	
Azotobacter	35			3·51	(−0·21)
Corynebacterium Vb	35			3·68	(−0·04)
		(*a*) 0·287	0·191	(*d*) 3·70	(−0·02)
,, Vb + *Azotobacter* ..	35	(*b*) 0·287	0·191	(*e*) 3·80	(0·08)
		(*c*) 0·288	0·190	(*f*) 3·51	(−0·21)
,, Vb ,, +10 c.c. H_2O	35			4·94	1·23

The data in Table 5 show clearly that under strictly aerobic conditions the efficiency of nitrogen fixation is very much lower than under the partly anaerobic conditions obtaining in the solution cultures: in series 1 (*Cor.* 3) it amounts to only about 2 mgm. N per gm. of decomposed cellulose, and in series 2 (*Cor.* Vb) it is altogether insignificant. Where the decomposing cellulose is immersed in a shallow layer of liquid, some nitrogen fixation takes place; abundant supply of moisture thus appears generally essential (cf. Jensen, 1940*a*).

Experiments 6–8: Next the utilization of natural cellulosic materials was tested. The first two series in Exp. 6 were carried out with finely ground and water-extracted wheat straw, 0·5 gm. in 100 c.c. round flasks with 0·2 gm. $CaCO_3$ and 40 c.c. basal solution + 1·0% yeast extract. Table 6 shows that with this material some nitrogen is also fixed, and the efficiency is about the same as with filter paper, but the actual amounts are much smaller. An additional experiment was carried out (Ser. III, Table 6) with a crude culture of obligate anaerobic cellulose-decomposing bacteria* in the same medium without yeast extract; this combination with *Azotobacter* gives a vigorous fixation, whereas *Cor.* 3 seems unable to induce fermentation in this medium which contains the N-compounds of the straw only. Experiment 7 was carried out as Exp. 6, in solution with 1·0% yeast extract, but with ground and water-extracted material of *Paspalum dilatatum*, harvested at the period of early seed formation. The results are given in Table 7, and show that *Azotobacter* + corynebacteria fix nitrogen with approximately the same efficiency as in straw, but in considerably larger amounts. Probably the cellulose in the grass-material is easier to digest than the more lignified straw-cellulose. The crude anaerobic culture again gives a very vigorous fixation. Experiment 8 was designed in a manner similar to Exp. 5 (decomposition under aerobic conditions): 0·5 gm. dry straw or *Paspalum*-material and 0·25 gm. $CaCO_3$, sterilized separately, were placed on the surface of 75 c.c. of agar medium in 250 c.c. round flasks; 1·0% yeast extract was added to the medium in the series in which straw was used. The results, summarized in Table 8, show no nitrogen fixation whatever under these conditions, even with extra addition of water.

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* Spore-bearing organisms of the type studied by Khouvine (1923); the culture was found to be free from aerobic cellulose-decomposers.

TABLE 6.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and Cellulose-decomposing Bacteria: Straw in Liquid Medium.*

Inoculum.	Incubation, Days.	Dry Organic Matter, gm. Per Culture.	Dry Organic Matter, gm. Loss.	Nitrogen, mgm. Per Culture.	Nitrogen, mgm. Gain.
I.					
Control	0	0·438		0·98	
Azotobacter	53			0·98	(0)
Corynebacterium 3	53			0·98	(0)
,, 3 + *Azotobacter* ..	53	0·391	0·047	1·20	0·22
II.					
Corynebacterium Vb	36	0·383	0·055	2·03*	
,, Vb + *Azotobacter* (triplicate)	36	0·370	0·068	2·71	0·68
III.					
Control (5 N-determinations) ..	0	0·438		0·78	
Azotobacter	21			0·78	(0)
Corynebacterium 3 + *Azotobacter* ..	21			0·72	(−0·06)
Crude anaerobic culture + *Azotobacter*	15	0·363	0·075	1·71	0·93
	33	0·336	0·102	2·02	1·24

Average gains of N, mgm. per gm. of dry matter lost:
Corynebacterium 3 + *Azotobacter* (series I): 4·7
,, Vb + ,, (series II): 10·0
Crude anaerobe + ,, 15 d. : 12·4
,, ,, + ,, 33 d. : 12·2

All figures averages of duplicates unless otherwise stated.

* Control analyses of this series lost; N-fixation therefore calculated as excess over pure culture of *Cor.* Vb.

Experiment 9: Nitrogen fixation with hemicellulosic material: In the experiments with straw and *Paspalum* it was thought that some fixation might be due to decomposition products of the hemicelluloses if these were attacked by the cellulose-decomposers. Most cellulose-decomposing fungi and actinomycetes can also attack hemicelluloses (Norman, 1937), but cellulose-decomposing bacteria have scarcely been studied in this respect; *Cytophaga*, according to Shrikande (1933), can decompose some of the hemicellulose in straw, and Verhulst *et al.* (1923) found strong decomposition of the hemicellulose in maize by *Cellulomonas flavigena*. Some tentative experiments were carried out with the organisms involved in the present study. Crude hemicellulose (xylan) was prepared from finely chopped wheat straw by extraction with hot 6% NaOH,

TABLE 7.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and Corynebacteria :* Paspalum-*material in Liquid Medium.*

Inoculum.	Incubation, Days.	Dry Matter, gm.		Nitrogen, mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Control	0	0·459		5·91 5·70 } 5·81	
Azotobacter	35			5·83	(0·02)
Corynebacterium 3	71	0·340	0·119	5·75	(−0·06)
,, Vb	71	0·340	0·119	5·94	(0·13)
,, 3+*Azotobacter* ..	40	(*a*) 0·306	0·153	(*c*) 7·38	1·57
		(*b*) 0·286	0·173	(*d*) 7·34	1·53
	72	(*a*) 0·250	0·209	(*a*) 5·68	(−0·13)
		(*b*) 0·261	0·198	(*b*) 7·59	1·78
,, Vb+*Azotobacter* ..	42	(*a*) 0·327	0·132	(*a*) 6·62	0·81
		(*b*) 0·342	0·116	(*b*) 6·71	0·90
	75	(*a*) 0·305	0·154	(*a*) 7·35	1·54
		(*b*) 0·311	0·148	(*b*) 6·92	1·11
Crude anaerobic cellulose-decomposers +*Azotobacter*	31			(*a*) 9·25	3·44
				(*b*) 9·09	3·24
Corynebacterium Va+*Azotobacter* ..	45	(*a*) 0·350	0·109	(*a*) 6·46	0·65
		(*b*) 0·355	0·104	(*b*) 6·50	0·69

Average gain of nitrogen per gm. of dry matter lost :
Corynebacterium Va+*Azotobacter*, 45 d. : 6·4 mgm.
,, Vb+ ,, 42 d. : 7·0 mgm.
,, Vb+ ,, 75 d. : 8·0 mgm.
,, 3+ ,, 40–72 d. : 9·4 mgm. (excluding the abnormal culture (*a*) after 72d.).

TABLE 8.

Nitrogen Content of Cultures of Azotobacter chroococcum *and Corynebacteria : Straw and* Paspalum-*material on Agar Surface.*

Inoculum.	Wheat Straw.		*Paspalum*-material.	
	Incubation, Days.	Total N, mgm.	Incubation, Days.	Total N, mgm.
Control	0	3·72 3·72 } 3·72	0	5·94 6·07 } 6·01
Corynebacterium 3	56	3·80	76	6·16
,, Vb	56	3·82	76	6·17
,, Vb+*Azotobacter*	35	(*a*) 3·80 (*b*) 3·63	75	(*a*) 6·23 (*b*) 6·13
,, Vb ,, +10 c.c. water ..	65	(*a*) 3·74 (*b*) 3·69	76	(*a*) 6·25 (*b*) 6·10
,, 3+*Azotobacter*	35	(*a*) 3·49 (*b*) —	75	(*a*) 6·28 (*b*) 6·26
,, 3 ,, +10 c.c. water ..	65	(*a*) 3·68 (*b*) 3·33 (?)	76	(*a*) 6·00 (*b*) 6·32

precipitation with alcohol, washing and drying. Qualitative tests showed that, with the exception of *Cytophaga*, all aerobic cellulose-decomposers previously examined (Jensen, 1940*b*) could attack this material; this was shown by the formation of enzymatic zones on agar and clearing of solution media, in which organic acids were formed by the corynebacteria, and reducing compounds (sugars?) by the other organisms. Three series of nitrogen-fixation experiments were conducted: (*a*) 0·4 gm. xylan, 0·2 gm. $CaCO_3$ and 20 c.c. of basal solution with 1·0% yeast extract in 50 c.c. round flasks; (*b*) similar, but xylan and $CaCO_3$ placed on surface of medium with 1% agar in 100 c.c. Erlenmeyer flasks; (*c*) as (*a*), but with 0·5 mgm. N as $(NH_4)_2SO_4$ instead of yeast extract. The corynebacteria and others demanding organic N were tested in the first and second, and the cellvibrios, actinomycetes and fungi in the third series. The results are given in Table 9.

TABLE 9.

Nitrogen Fixation in Combined Cultures of Azotobacter chroococcum *and Cellulose-decomposing Bacteria, in Crude Hemicellulose.*

Inoculum.	In Solution.			On Agar Surface.		
	Incubation, Days.	Total N, mgm.		Incubation, Days.	Total N, mgm.	
		Per Culture.	Gain.		Per Culture.	Gain
Control	0	1·16		0	1·30	
Azotobacter (1)	20	1·39	(0·23)			
,, (1)	42	0·99	(−0·17)	48	1·22	(−0·08)
Corynebacterium 3 (1)	46	0·94	(−0·22)	48	1·29	(−0·01)
,, Vb (1)	46	1·19	(0·03)	48	1·26	(−0·04)
,, 3 + *Azotobacter*	20	1·55	0·39			
,, 3 + ,,	46	4·07	2·91	48	1·88	0·58
,, Vb + ,,	20	1·70	0·54			
,, Vb + ,,	46	2·64	1·48	48	1·56	0·26
,, Va + ,,	42	2·87	1·71	46	2·00	0·70
Cellulomonas biazotea + *Azotobacter*	42	3·50	2·34	46	2·26	0·96
Bacterium 43 + *Azotobacter*	35	1·14	(−0·02)			
,, G +	35	1·19	(0·03)			

Third series: in $(NH_4)_2SO_4$-solution.

Inoculum.	Incubation, Days.	Total N, mgm.	Inoculum.	Incubation, Days.	Total N, mgm.
Control	0	0·57	*Cellvibrio* G3 + *Azotobacter*	42	0·60
Actinomyces sp.	43	0·54	,, 17 + ,,	42	0·59
Cellvibrio G2	43	0·63	*Actinomyces* + ,,	43	0·61
,, G2 + *Azotobacter*	42	0·59	*Micromonospora* + ,,	43	0·57
,, G1 +	42	0·63	*Trichoderma* +	43	0·58

Azotobacter together with the corynebacteria gives a vigorous fixation, and *Cell. biazotea*, so little active in cellulose-media, appears remarkably effective here. Since the organic matter of the impure xylan was not all used up, it is obvious that the efficiency of fixation in cultures of *Cor.* 3 and *Cell. biazotea* exceeds 9–12 mgm. N per gm. of material decomposed. On the agar surface some nitrogen is also fixed, but the amount is only roughly one-sixth to one-third of that in solution (cf. Exp. 5). The cellulose-decomposing spore-formers *Bac.* G and *Bac.* 43, which were previously found unable to feed *Azotobacter* from cellulose (Jensen, 1940*b*), behave likewise with the xylan. In the cultures of cellvibrios, actinomycetes and fungi there is no trace of nitrogen fixation, and no growth of *Azotobacter* was seen.

B. *Nitrogen Fixation in Culture Filtrates.*

In the associated cultures *Azotobacter* might derive energy from the cellulose either by intercepting hydrolysis products of cellulose before these were taken up by the corynebacteria, or by utilizing the organic acids and possibly other organic by-products of these latter organisms, or finally in both ways. In order to test the value of the by-products as nutrients for *Azotobacter*, this organism was grown in filtrates from cultures of the cellulose-decomposers.

In the first experiment, *Cor.* 3 was grown for 48 days at 28–30°C. in 2·0% yeast-extract solution with filter paper and $CaCO_3$ (cf. Ser. I, Table 1), and *Cor.* Vb. was grown for 45 days at 37°C. in the same medium. After filtration through glass crucibles and determination of the loss of cellulose, the filtrates were made up to definite volumes. After addition of 0·01% $FeCl_3$, 0·001% Na_2MoO_4 and 0·2% $CaCO_3$, the filtrates were divided into portions of 50 c.c., which were distributed in 250 c.c. Erlenmeyer flasks, sterilized, inoculated with *Az. chroococcum*, and incubated at 28–30°C. A control experiment was run with *Cor.* Vb in yeast-extract solution without filter paper. The results are set out in Table 10.

TABLE 10.

Nitrogen Fixation by Azotobacter chroococcum *in Culture Filtrates of* Corynebacterium *3 and* Corynebacterium *Vb.*

Source of Filtrate.	Incubation, Days.	Total N, mgm.	Gain of N, mgm.	
			Per Culture.	Per gm. of Cellulose Decomposed.
I.				
Corynebacterium 3, 48 d., 28–30° C.	0	0·78 0·72 } 0·75		
Each culture ≡ 0·057 gm. decomposed cellulose*	6	(*a*) 0·92	0·17	3·0
		(*b*) 1·00	0·25	4·4 } *3·7*
		(*c*) 0·96	0·21	3·7
II.				
Corynebacterium Vb, 45 d., 37° C.	0	2·71 2·64 } 2·68		
	8	(*a*) 3·13	0·45	2·3 } *2·2*
Each culture ≡ 0·193 gm. decomposed cellulose*		(*b*) 3·09	0·41	2·2
	15	(*a*) 3·55	0·87	4·5 } *4·7*
		(*b*) 3·64	0·96	5·0
III.				
As II, +0·5% glucose	15	(*a*) 5·45	2·77	
		(*b*) 5·34	2·66	
IV.				
Corynebacterium Vb, 45 d., 37° C.	0	2·88 2·80 } 2·84		
Cellulose-free control medium	14	(*a*) 2·80	(−0·04)	
		(*b*) 2·78	(−0·06)	

* Calculated on the basis of the total amount of cellulose decomposed.

In all filtrates from cellulose cultures, *Azotobacter* grew well, and the gains of nitrogen are significant; no nutrients for *Azotobacter* are produced from the yeast extract itself. Glucose, when added to the medium, is utilized with an approximately normal efficiency. Evidently the nitrogen compounds present in the filtrate, chiefly as bacterial cells, do not interfere with the fixation, nor does the filtrate appear to contain special stimulating factors (cf. Greaves *et al.* (1940), who claim to have observed a strong stimulative effect on *Azotobacter* by small amounts of amino acids, etc.).

In the main experiment the amount of soluble carbon in the filtrate was determined, and an attempt was made to estimate the nutritive value of its volatile and non-volatile constituents. *Cor.* Vb was grown in ten replicate cultures in 250 c.c. Erlenmeyer flasks with 1·0 gm. filter paper, 1·0 gm. $CaCO_3$, and 100 c.c. solution + 4·0% yeast extract. After incubation for 25 days at 37°C. the cultures were filtered through glass crucibles; the total loss of cellulose was 3·35 gm. The combined filtrates and washings with distilled water were made up to 1000 c.c. and treated according to the following scheme:

A. ("Original filtrate").—200 c.c. of filtrate were used for carbon determination, etc.; reducing sugars as well as neutral volatile compounds (alcohol) were found to be absent. To another 300 c.c. of filtrate were added 0·05% K_2HPO_4, 0·01% $FeCl_3$, 0·1%

$CaCO_3$, and 0·001% Na_2MoO_4; the medium was then distributed to 250 c.c. Erlenmeyer flasks in six portions of 50 c.c., each of which thus represented the total decomposition products of 0·167 gm. cellulose. The flasks were sterilized, inoculated with *Az. chroococcum*, and incubated at 28–30°C.

B. ("Volatile fraction").—The volatile compounds were removed from 500 c.c. of original filtrate by steam distillation after acidification with sulphuric acid. The distillate was made up to 600 c.c., of which 100 c.c. were used for carbon determination; the remaining 500 c.c. were neutralized with a small excess of $CaCO_3$, reduced to about 200 c.c. on a water bath, made up to 240 c.c. and given additions of 0·05% K_2HPO_4, 0·02% $MgSO_4$, 0·02% NaCl, 0·01% $FeCl_3$, and 0·001% Na_2MoO_4. This medium was again distributed to 250 c.c. Erlenmeyer flasks in six portions of 40 c.c., each of which represented the volatile decomposition products of 0·233 gm. cellulose. The flasks were sterilized, inoculated with *Azotobacter*, and incubated, as under (A).

C. ("Non-volatile fraction").—The residue after steam distillation of 500 c.c. of original filtrate under (B) was neutralized with $CaCO_3$, filtered and made up to 400 c.c., of which 100 c.c. were used for carbon determination, etc. (no reducing sugar). To the remaining 300 c.c. were added 0·05% K_2HPO_4, 0·02% $MgSO_4$, 0·01% $FeCl_3$, 0·001%

TABLE 11.

Nitrogen Fixation by Azotobacter chroococcum *in Culture Filtrate, and Fractions Thereof, from* Corynebacterium *Vb*$_4$

Culture Series.	Incubation, Days.	Total N, mgm.	Gain of N. mgm.		
			Per Culture.	Per gm. Carbon in Culture.	Per gm. Cellulose Decomposed.
I. Original filtrate; each culture≡0·167 gm. decomposed cellulose.	0	2·90 2·84 } 2·87			
	10	(*a*) 3·58	0·71	29	4·2
		(*b*) lost	—	—	—
Total organic carbon at start:	16	(*a*) 3·67	0·80	33	4·8
25·3 23·0 } *24·2* mgm. per culture		(*b*) 3·67	0·80	33	4·8
II. Volatile fraction; each culture≡0·233 gm. decomposed cellulose.	0	0·11 0·08 } 0·10			
	10	(*a*) 0·32	0·22	19	0·9
Total organic carbon per culture at start:		(*b*) 0·28	0·18	16	0·8
	16	(*a*) 0·33	0·23	20	1·0
10·7 12·0 } *11·4* mgm.		(*b*) 0·24	0·14	12	0·6
III. Non-volatile fraction; each culture≡0·209 gm. decomposed cellulose.	0	3·18 3·19 } 3·19			
	10	(*a*) 3·97	0·78	38	3·7
Total organic carbon per culture at start:		(*b*) 3·81	0·62	30	3·0
		(*a*) 3·90	0·71	34	3·4
20·7 20·7 } *20·7* mgm.		(*b*) 3·86	0·67	32	3·2
IV. Filtrate from crude culture of anaerobic cellulose decomposers. Each culture≡0·137 gm. decomposed cellulose.	0	0·71 0·71 } 0·71			
	7	(*a*) 1·38	0·67	42	4·9
		(*b*) 1·20	0·49	30	3·6
Total organic carbon per culture at start:	14	(*a*) 1·33	0·62	38	4·5
16·9 16·0 15·4 } *16·1* mgm.		(*b*) 1·52	0·81	50	5·9
Do., sterile control	14	0·80	(0·09)	—	—

Na_2MoO_4, and 0·1% $CaCO_3$. The medium was then divided into six portions of 50 c.c., each representing the non-volatile decomposition products of 0·209 gm. cellulose. Erlenmeyer flasks were sterilized, inoculated and incubated as before.

The results of the nitrogen-fixation experiments are seen in Table 11, which includes a series with filtrate from a crude culture of anaerobic cellulose-decomposing bacteria: 2 × 250 c.c. basal solution with 2·0 gm. filter paper, 0·5 gm. $CaCO_3$, and 0·1 gm. dry matter of *Azotobacter* as source of N. Cultures were incubated 59 days at 37°C.; 2·05 gm. cellulose had been decomposed. The filtrate was treated as under (A).

The data show that *Azotobacter* utilizes the carbon compounds of the original filtrate very economically, fixing more than 30 mgm. N per gm. of C present, or close to 5 mgm. per gm. of cellulose decomposed (cf. Table 10). Of this total fixation only a minor part appears to be due to the volatile decomposition products, which are also of lesser value per unit of carbon. The bulk of the fixation (3·0–3·7 mgm. N per gm. cellulose) takes place at the expense of the non-volatile products, which appear to be good nutrients, some 30 to 40 mgm. N being fixed per gm. C present; this is similar to the gain in ordinary *Azotobacter*-cultures fixing 12 to 16 mgm. N per gm. of glucose. The decomposition products of the anaerobic cellulose-decomposers seem to be at least equally valuable.

As mentioned above, reducing sugars or substances giving rise to such compounds on acid-hydrolysis were not present in the culture filtrates. A control experiment was therefore made to test the nutritive values of various organic acids in concentrations similar to the carbon content of the filtrates and their fractions. This experiment is recorded in Table 12. The culture medium consisted of 50 c.c. basal solution in 250 c.c. Erlenmeyer flasks, with a trace of $CaCO_3$ and calculated amounts of organic acids as Ca-salts.

TABLE 12.

Nitrogen Fixation by Azotobacter chroococcum *in Dilute Solutions of Organic Acids.*

Series.	Total N per Culture, mgm.*	Gain of N, mgm.		
		Per Culture.	Per gm. Org. C.	Per gm. Acid.
I. 25 mgm. C as formic acid.				
Control	0·03	—	—	—
Azotobacter inc. 14 d.	0·05	(0·02)	—	—
II. 25 mgm. C as acetic acid.				
Control	0·05	—	—	—
Azotobacter inc. 12 d.	0·71	0·66	26·6	10·6
III. 50 mgm. C as acetic acid.				
Control	0·02	—	—	—
Azotobacter inc. 17 d.	0·80	0·78	15·8	6·3
IV. 25 mgm. C as lactic acid.				
Control	0·04	—	—	—
Azotobacter inc. 12 d.	0·84	0·80	32·0	12·8
V. 25 mgm. C as gluconic acid.				
Control	0·07	—	—	—
Azotobacter inc. 8 d.	0·73	0·66	26·6	9·8
,, ,, 14 d.	0·79	0·72	27·8	10·8

* Averages of duplicates.

Formic acid is not utilized (cf. Gainey, 1928), but acetic acid, especially in the lower concentration, is a favourable source of carbon; the fixation of about 26 mgm. N per gm. acetate-C is consistent with the gain of 12–20 mgm. per gm. C in the mixture of formic and acetic acid which represents the "volatile fraction" in Table 11. Lactic acid is an even better nutrient (cf. Mockeridge, 1915); the fixation of 32 mgm. N per gm. lactate-C agrees with the corresponding figures for "original filtrate" and "non-volatile fraction" in Table 11. Finally, gluconic acid appears to be only a moderately good source of carbon. So far the results suggest that the bulk of the non-volatile constituents is represented by substances not identical with reducing sugars, but similar or superior to lactic acid in nutritive value.*

C. *Influence of Aeration on the Formation of Organic By-products.*

The slight or negligible nitrogen fixation in cultures with cellulosic material in direct contact with the air (Tables 5, 8, and 9) suggests that the organic by-products under these conditions are formed in smaller amounts or consist of less valuable nutrients (formic acid?). An experiment was designed to find the quantities of total free acid produced from cellulose under varying conditions of aeration. *Cor.* 3 and *Cor.* Vb were grown in 50 c.c. of basal solution with 2·5% yeast extract and 0·5 gm. filter paper, but no $CaCO_3$: (*a*) in 100 c.c. round flasks, the paper thus submerged in a layer of liquid about 2·5 cm. deep; (*b*) in 250 c.c. Erlenmeyer flasks, layer of liquid about 1 cm. deep, and (*c*) as (*b*), but paper resting on a raft of glass tubes of 1 cm. diameter, thus being in contact with the atmosphere. Cultures were incubated at 28–30°C., then filtered through glass crucibles; loss of cellulose was determined, and the filtrates were titrated with approximately 0·035 n NaOH to the same reaction (pH about 7·5) as filtrate from the sterile control medium. The results are given in Table 13.

TABLE 13.

Influence of Access of Oxygen on Production of Acid from Cellulose by Corynebacteria.

	Method of Placing Filter Paper.		Cellulose Lost, gm.	Total Free Acid, c.c. 0·1n.	
				Per Culture.	Per gm. Cellulose Lost.
Corynebacterium 3 (triplicate) inc. 26 d.	Deep layer		0·048	1·49	31·0
	Shallow layer		0·045	0·98	21·8
	In contact with air		0·062	0·85	13·7
Corynebacterium Vb (duplicate).	Deep layer	22 d.	0·032	1·29	40·3
		42 d.	0·042	1·58	37·6
	Shallow layer ..	22 d.	0·041	1·23	30·0
		42 d.	0·064	0·99	15·5
	In contact with air ..	22 d.	0·090	0·98	10·9
		42 d.	0·127	1·15	9·1

This simple experiment shows clearly that the formation of acid in proportion to cellulose decomposition falls off markedly with an increasing degree of aeration, especially in *Cor.* Vb. It is interesting to compare this with the results of nitrogen-fixation experiments on agar surfaces (Table 5), where *Cor.* 3 caused a slight fixation, but *Cor.* Vb none.

Another experiment was designed to test the nutritive value of the by-products formed under complete or restricted aeration. *Cor.* Vb was grown in two series, each of five replicate cultures; the medium consisted of 1·0 gm. filter paper, 0·5 gm. $CaCO_3$, and 60 c.c. basal solution with 3·0% yeast extract. Five cultures were grown in 100 c.c. round flasks, and five in 500 c.c. Erlenmeyer flasks, the paper resting on a raft of glass tubes as above. After incubation for 20 days at 37°C. the cultures were filtered, and loss of cellulose determined: 1·23 gm. in the five round flasks, and 0·60 gm. in the five Erlenmeyer flasks. The combined filtrates and washings from each series, before HCl-treatment, were reduced in volume on the water bath, and then made up to

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* It should not be forgotten that some of the carbon in the culture filtrates is represented by proteid or related matter (bacterial cells, residues from the yeast extract, etc.). If this, as is probable, remains inaccessible to *Azotobacter*, the gain of nitrogen per unit of *available* carbon would be considerably higher than it appears. The amount of available carbon cannot be readily ascertained, but if for each part of N in the medium we allow for three parts of protein-C, the gains of nitrogen per gm. of "non-protein carbon" in Series I, III and IV of Table 11 would be in the neighbourhood of 50-60 mgm.—a very high degree of economy.

300 c.c., to which were added 0·05% K_2HPO_4, 0·01% $FeCl_3$, 0·001% Na_2MoO_4, and 0·1% $CaCO_3$. Each lot of medium was divided into six portions of 50 c.c.; one of these was used for carbon determination, and the rest were placed in 250 c.c. Erlenmeyer flasks, sterilized, inoculated with *Az. chroococcum*, and incubated. Table 14 gives the results.

TABLE 14.

Influence of Access of Oxygen on Production of Food Substances for Azotobacter *by* Corynebacterium *Vb.*

Culture Series.	Incubation, Days.	Total N in *Azotobacter* Cultures.	Gain of N, mgm.		
			Per Culture.	Per gm. Carbon in Culture.	Per gm. Cellulose Decomposed.
Paper in contact with atmosphere.	0	2·19 2·21 } 2·20	—	—	—
Each culture ≡ 0·100 gm. decomposed cellulose.	10	2·36	(0·16)	(7·0)	(1·6)
Total organic carbon at start: 25·2 20·5 } 22·8 mgm.	14	2·46 2·36 } 2·41	(0·21)	(9·2)	(2·1)
Paper submerged in solution. Each culture ≡ 0·205 gm. decomposed cellulose.	0	2·11 2·22 } 2·16	—	—	—
Total organic carbon at start:	10	3·30	1·14	22	5·5
53·5 51·0 } 52·3 mgm.	14	3·44 3·24 } 3·34	1·18	23	5·7

The filtrate from the well-aerated Erlenmeyer flask cultures is seen to be very poor in soluble carbon compounds, and these are only inferior nutrients for *Azotobacter*; indeed, the gain of nitrogen is so slight as to be of questionable significance. The filtrate from cellulose decomposed under restricted aeration in round flasks contains a much larger amount of organic matter, which also permits a stronger nitrogen fixation per unit of carbon present. It seems clear that not only does restricted access of oxygen result in the formation of larger quantities of organic by-products, but these are also qualitatively better nutrients for *Azotobacter*.

The nitrogen fixation by *Azotobacter* in culture filtrates from the cellulose-decomposers is seen generally to be of the order of about 4 to 6 mgm. N per gm. of cellulose decomposed, i.e., about one-half of that which is found in associated cultures. Two explanations might be offered for the fact that more nitrogen is fixed when the two organisms work in association: *Azotobacter* might have access to intermediate hydrolysis products of the cellulose, or the oxygen tension in the medium might be lowered by the growth of the aerobic *Azotobacter* and the cellulose-decomposers thus be induced to form metabolic by-products in larger quantity and of higher value for *Azotobacter* than they would in pure culture. If the second possibility holds, one would expect the oxidation-reduction potential of the medium to be lower in associated cultures than in pure cultures of the cellulose-decomposers (cf. Vartiovaara, 1938, who observed only a slight decrease in the oxidation-reduction potential in cultures of cellulose-decomposing fungi, but very strong reduction in cultures of fungi + nitrogen-fixing clostridia). An attempt was made to find out if this is the case.

Cor. Vb was grown, alone or together with *Az. chroococcum*, in basal solution with 1·0% yeast extract, $CaCO_3$ and strips of filter paper—(*a*) in a deep column of liquid, viz., 10 c.c. of medium in 1·2 cm. wide test tubes—and (*b*) a more shallow layer allowing better aeration: 5 c.c. of medium in test tubes 2 cm. wide. Cultures and sterile controls were incubated at 28–30°C.; at intervals, duplicate tubes were taken out and the oxidation-reduction potential (Eh) measured electrometrically, using a bright platinum foil electrode, with a 3·5 n KCl-HgCl-electrode as standard half-cell. (On oxidation-reduction potentials in general and their application to bacteriological problems, see Hewitt, 1936.) After Eh-measurement, the pH-value of each tube was determined by means of a glass electrode. The results, averaged and corrected to pH 7·0, are shown graphically in Text-figure 1. The conventional factor of 0·070 volt per unit pH was used for the correction; it is to be remembered that in oxidation-reduction systems of unknown nature this factor has only an approximate meaning (Hewitt, 1936).

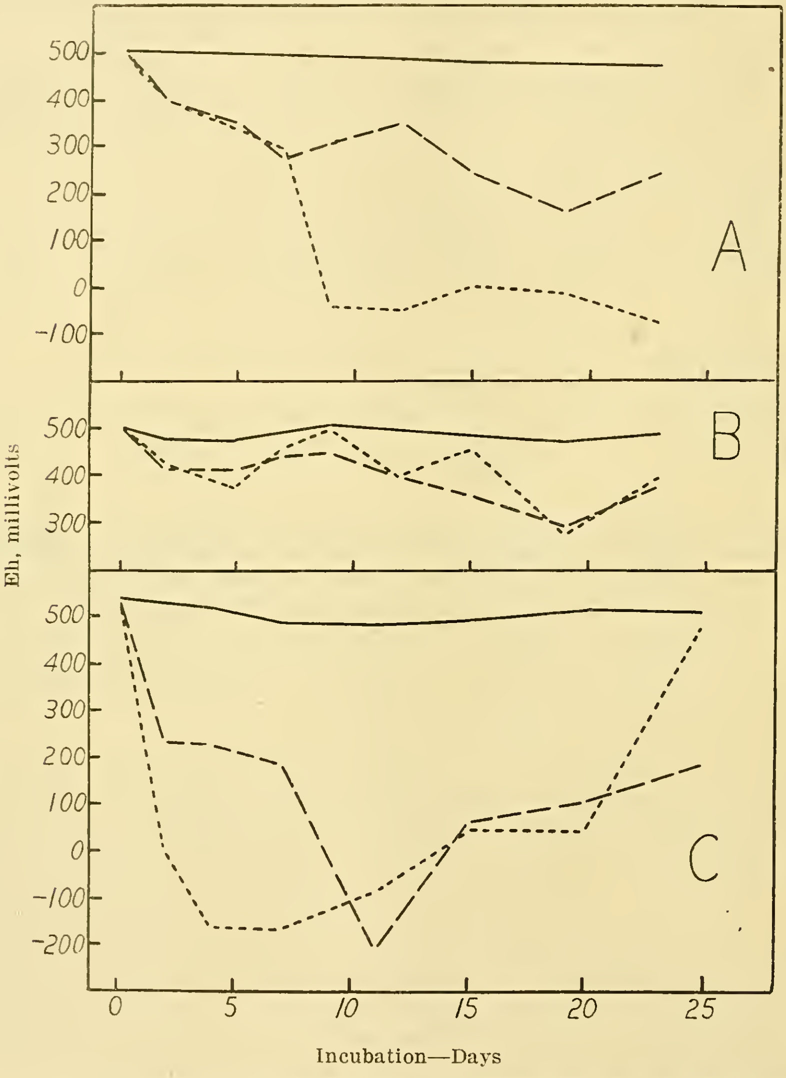

Fig. 1.—Oxidation-reduction potentials (Eh) in pure and combined cultures. A, filter paper in a deep layer of medium (10 c.c.). B, filter paper in a shallow layer of medium (5 c.c.). C, hemicellulose. Continuous lines: sterile medium; broken lines: *Corynebacterium* Vb; dotted lines: *Cor.* Vb + *Az. chroococcum.*

The graph shows that the Eh-values of the sterile medium remain practically stationary, and *Cor.* Vb alone causes only a moderate decrease. In the associated cultures in the deep layer of medium, *Azotobacter* started to make a good growth after one week; at the same time the Eh-values showed a sudden decline, and remained from then on at the level characteristic of most aerobic bacteria (Hewitt, 1936). In the shallow layer of medium (wide tubes) the growth of *Azotobacter* was scant, and no effect on Eh was noticeable.

A similar experiment, shown in Figure 1c, was carried out in solution of 0·5% hemicellulose (crude xylan), in deep layers of medium only. The corynebacterium grows more rapidly in this medium than on cellulose, and the fall in Eh of pure cultures is quite pronounced, reaching a very low minimum after 11 days, but is much more rapid and persistent in the presence of *Azotobacter.*

Upon the whole, the experiments indicate clearly that under limited access of oxygen (deep layers of solution) *Azotobacter* is capable of creating conditions favourable for the production of organic compounds from the cellulose, and this is probably why nitrogen fixation is stronger in associated cultures than when the organisms exist in "metabiosis" (*Azotobacter* in culture filtrates). It is of course not excluded that intermediate products may also be used, but this seems unlikely, since reducing sugars can never be detected in pure cultures of the corynebacteria, even when deprived of oxygen, and since the nitrogen fixation is so slight in cellulosic material directly exposed to the air, where the conditions for interception by *Azotobacter* should be the best possible.

D. *The Decomposition Products of Cellulose.*

An attempt was made to identify the decomposition products in pure cultures of the cellulose-decomposers, especially *Cor.* Vb. The determination of the volatile products presented no difficulties. Ethyl alcohol and other neutral volatile compounds were not found; among acidic compounds, only formic and acetic acid in varying proportions were detected; a summary of the main results is given in Table 15. In cultures with ample supply of nitrogen (peptone) formic acid is the chief volatile product, which in *Cor.* 3 accounts for no less than 60% of the carbon in the decomposed cellulose. Formic acid was also the only volatile product in other cultures of *Cor.* Vb with straw and glucose.

TABLE 15.

Decomposition Products of Cellulose.

Experiment Number.	Loss of Cellulose, gm.	Decomposition Product Found.	% C in Decomposed Cellulose Accounted for.	
I. *Corynebacterium* Vb, 15 gm. filter paper; solution of 2·5% yeast extract. Inc. 90 d. 28–30° C.	1·22	Total volatile acid (partly formic), 6·5 c.c. 1/n, containing 0·077 gm. C.	14·3	
II. *Corynebacterium* Vb, 18 gm. filter paper; solution of 1% yeast extract, 0·5% peptone. Inc. 12 d. 28–30° C.	7·75	Volatile acids, formic and acetic, containing 0·710 gm. C.	20·3	
III. *Corynebacterium* Vb, 6 gm. filter paper; solution of 1% yeast extract, 0·5% peptone. Inc. 20 d. 37° C.	4·38	Formic acid, 1·65 gm.	22·2	31·2
		Acetic acid, 0·341 gm.	7·0	
		Lactic acid, 0·098 gm.	2·0	
IV. *Corynebacterium* 3, 5 gm. filter paper; solution of 1% yeast extract, 0·2% peptone. Inc. 24 d. 37° C.	1·84	Formic acid, 1·91 gm.	60·4	62·9
		(Acetic acid, nil)	(0)	
		Lactic acid, 0·050 gm.	2·5	

The non-volatile products, on the other hand, proved most difficult to determine, because of the necessity of adding yeast extract and peptone to the medium in order to obtain a vigorous decomposition. In Exp. I, Table 15, the calcium salts of the non-volatile acids other than lactic acid were precipitated with 80% alcohol according to the method of Birkinshaw and Raistrick (1931). Most of the total carbon in the culture filtrate was found in this precipitate, as shown below:

Carbon represented by 1·22 gm. decomposed cellulose	0·536 gm.
Total carbon found in culture filtrate	0·427 gm.
Loss (probably CO_2)	0·109 gm.
Analysis of filtrate:	
Carbon recovered as volatile acids	0·077 gm.
Carbon recovered as non-volatile acids (alcohol-precipitate)	0·362 gm.
Carbon recovered as residual compounds (lactic acid?)	0·072 gm.
Total	0·421 gm.

The precipitate of calcium salts contained no compounds reducing Fehling's solution (ketonic and aldehydic acids absent), but decolourized $KMnO_4$ and gave slight reduction of ammoniacal $AgNO_3$; oxalic and tartaric acids were not found; the iodoform test for citric acid was doubtful. The experiment thus suggests that most of the food for *Azotobacter* consists of unidentified organic acids; the calcium salts of these must be relatively soluble, since no precipitate was formed on addition of calcium acetate before alcohol was added.

In the solutions containing peptone the method of Birkinshaw and Raistrick (1931) proved unsuccessful, and gave precipitates consisting mainly of peptone or amino acids. It was found that the direct determination of lactic acid in the culture filtrates, using the method of oxidation to acetaldehyde with $KMnO_4$, was vitiated by the presence of other compounds that also yielded aldehydes. Small quantities of lactic acid were found in ether-extracts of acidified dry residues of the filtrates (Table 15). Carbon determinations in culture filtrates from Exp. II and III, however, showed a greater content of soluble carbon than could be represented by the sum of the volatile acids, lactic acid, and peptone. Thus there must be other compounds present, but since no reduction of Fehling's solution was found, these could not include cellobiose, glucose, aldehydic or ketonic acids. The possible formation of gluconic acid was thought of, and therefore an attempt was made to isolate this compound from cultures of *Cor.* Vb by the method of Müller (1928): precipitation with lead subacetate in ammoniacal solution, decomposition with H_2S-gas, conversion into Ca-salt, and crystallization from 30% alcohol. Only flocculent precipitates of a proteid-like appearance were found in the final stage, and in one case a precipitate resembling a mixture of proteids and calcium formate. Like the cellulose-decomposing bacteria of Khouvine (1923) and Simola (1931), the corynebacteria thus seem to produce compounds that cannot readily be identified. Finally it may be mentioned that a trace of reducing sugar was found in a culture of *Cor.* Vb, treated with toluene and incubated 4 weeks at 37°C., 1·74 gm. cellulose being decomposed; the sugar yielded an osazone resembling that of glucose (long needles, melting point 206°C.).

In general it appears that the formation of metabolic products varies greatly with the experimental conditions, and that the non-volatile compounds which represent the best sources of carbon for *Azotobacter*, are formed chiefly in media with a low nitrogen content, such as in Exp. I, Table 15. The associated cultures exist in such media poor in nitrogen, and therefore the metabolism of the cellulose-decomposers is under these conditions probably quite different from that in pure cultures with abundant supply of nitrogen. This is borne out by the fact that up to 14 mgm. N may be fixed per gm. of cellulose decomposed (Table 1); assuming that *Azotobacter* may fix 1 part of nitrogen per 20 parts of carbon consumed (the maximal efficiency observed according to Table 11), such a fixation would require the consumption of 280 mgm. carbon—i.e., nearly two-thirds of the cellulose-carbon must be converted into nutrients favourable for *Azotobacter*, and hence the production of compounds like carbon dioxide and formic acid must be very low.

General Conclusions.

Some conclusions of general interest may be drawn from these data. Firstly it appears that the fixation of some 10 mgm. nitrogen per gm. of cellulosic material decomposed, which has so frequently been observed in impure cultures or in soil, may be due to the activity of cellulose-decomposing corynebacteria in co-operation with *Azotobacter*. Secondly, it was found (Jensen, 1940*a*) that nitrogen fixation on the basis of cellulosic materials like straw, etc., only takes place in soil of high moisture content. This phenomenon finds a natural explanation in the fact that a limited access of oxygen is necessary for a copious formation of organic by-products by the corynebacteria; it is also a fact that exclusion of oxygen is necessary for development of the obligate anaerobic cellulose-decomposers as well as for production of soluble organic compounds by the true aerobic cellulose-decomposers (cellvibrios, fungi, etc.).

As to the agricultural significance of the association between *Azotobacter* and cellulose-decomposing corynebacteria, it would seem that arable soils under the climatic conditions obtaining in Australia would rarely offer a favourable environment, even if generally favourable for *Azotobacter*, as previously discussed (Jensen, 1940*a*). The process may be of high importance under conditions where prolonged partial exclusion of oxygen coincides with a favourable temperature, such as in compost or manure heaps, or irrigated land rich in organic matter of a wide C/N ratio. Cellulosic waste materials might therefore through the activity of these organisms be used as a profitable source of nitrogenous manure.

SUMMARY.

Combinations of pure cultures of various species of *Azotobacter* and cellulose-decomposing corynebacteria can fix about 6 to 14 mgm. nitrogen per gm. of cellulose decomposed. Filter paper, grass and straw can be utilized with approximately the same efficiency, but the last material less rapidly. Hemicellulose, in the form of crude xylan, can also be utilized with a high efficiency.

Nitrogen fixation of this magnitude was observed only when the cellulosic material was submerged in liquid medium. Under strictly aerobic conditions (decomposing material in direct contact with the air), the fixation was less, or negligible (0 to 2 mgm. per gm.).

Azotobacter was able to grow in filtrates from cellulose-cultures of the corynebacteria, and to fix as much as 5·7 mgm. nitrogen at the expense of the decomposition products of each gm. of cellulose; most of the fixation took place by utilization of the non-volatile decomposition products. Under completely aerobic conditions the quantity of organic decomposition products of cellulose, as well as the value of these compounds as nutrients for *Azotobacter*, was less than under restricted access of oxygen.

The oxidation-reduction potential in combined cultures of *Azotobacter* and corynebacteria in cellulose-media reached a considerably lower level than in pure cultures of the corynebacteria, provided that a deep layer of liquid medium was used.

In pure cultures of the corynebacteria, the volatile decomposition products of cellulose consisted exclusively of formic and acetic acid in varying proportions, the formic acid accounting for as much as 60% of the carbon in the decomposed cellulose. Small amounts of lactic acid were found among the non-volatile products, besides certain not readily identifiable compounds, probably organic acids. Reducing sugars, ketonic acids and aldehydic acids were absent in normal cultures, but traces of glucose were detected in cultures treated with toluene.

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